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(57) Abstract

The present invention provides new mononucleotide synthons useful in the synthesis of oligonucleotides having from one t all P-chiral centers that are predominantly and independently in the R or S configuration. The invention also provides methods of synthesizing these synthons, methods of synthesizing oligonucleotides having from one to all P-chiral centers predominantly and independently in the R or S configuration, and such oligonucleotides. Oligonucleotides synthesized with the novel synthons are useful for modulating nucleic acid expression, both *in vitro* and *in vivo*, as well as in traditional hybridization assays.

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METHODS AND COMPOUNDS FOR THE SYNTHESIS OF OLIGONUCLEOTIDES AND THE OLIGONUCLEOTIDES THEREBY PRODUCED

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to methods for the chemical synthesis of oligonucleotides, compounds useful in the methods, and oligonucleotides thereby produced.

Description of the Prior Art

Oligonucleotides and their chemical synthesis have become indespensible tools in modern molecular biology, being used in a wide variety of techniques ranging from PCR to antisense inhibition of nucleic acid expression. Understandably, therefore, there has been an every increasing demand for oligonucleotides having desirable properties such as resistance to nucleolytic attack and increased binding affinities. Furthermore, with the widespread use of oligonucleotides for these varying purposes, there has been an ever increasing demand for fast, inexpensive, and efficient methods of synthesizing oligonucleotides having these desirable properties.

Since Zamecnik and Stephenson, *Proc. Natl. Acad Sci. USA* **75**, 280-284 (1978), first demonstrated virus replication inhibition by synthetic oligonucleotides, there has been much interest in the use of antisense oligonucleotides as agents for the selective modulation of gene expression, both *in vitro* and *in vivo*. *See, e.g.*, Agrawal, *Trends in Biotech.* **10**, 152 (1992); Chang and Petit, *Prog. Biophys. Molec. Biol.* **58**, 225 (1992); and Uhimann and Peymann, *Chem. Rev.* **90**, 543 (1990). Antisense oligonucleotides are

constructed to be sufficiently complementary to a target nucleic acid to hybridize with the target under the conditions of interest and inhibit expression of the target. Antisense oligonucleotides may be designed to bind directly to DNA (the so-called "anti-gene" approach) or to viral RNA or mRNA. *Id.* Expression inhibition is believed to occur by interfering with transcription processing or translation, or inducement of target mRNA cleavage by RNase H.

Antisense oligonucleotides can be used as research tools *in vitro* to determine the biological function of genes and proteins. They provide an easily used alternative to the laborious method of gene mutation (*e.g.*, deletion mutation) to selectively inhibit gene expression. The importance of this method is readily appreciated when one realizes that the elucidation of most known biological processes has been determined by deletion mutation.

Antisense oligonucleotides also may be used to treat a variety of pathogenic diseases by inhibiting gene expression of the pathogen *in vivo*. Oligonucleotide phosphorothioates (PS-oligos) have shown great therapeutic potential as antisense-mediated inhibitors of gene expression (Stein and Cheng, *Science* **261**, 1004 (1993) and references therein) as evidenced by a number of ongoing clinical trials against AIDS and cancer. Agrawal and Tang, *Antisense Res. and Dev.* **2**, 261 (1992) and references therein, and Bayever et al., *Antisense Res. Dev.* **3**, 383 (1993).

The synthesis of oligonucleotides for antisense and diagnostic applications is now be routinely accomplished. ***Methods in Molecular Biology, Vol 20: Protocols for***

Oligonucleotides and Analogs pp. 165-189 (S. Agrawal, Ed., Humana Press, 1993); *Oligonucleotides and Analogues: A Practical Approach*, pp. 87-108 (F. Eckstein, Ed., 1991); and Uhlmann and Peyman, *supra*. Agrawal and Iyer, *Curr. Op. in Biotech.* 6, 12 (1995); and *Antisense Research and Applications* (Crooke and Lebleu, Eds., CRC Press, Boca Raton, 1993). Early synthetic approaches included phosphodiester and phosphotriester chemistries. Khorana et al., *J. Molec. Biol.* 72, 209 (1972) discloses phosphodiester chemistry for oligonucleotide synthesis. Reese, *Tetrahedron Lett.* 34, 3143-3179 (1978), discloses phosphotriester chemistry for synthesis of oligonucleotides and polynucleotides. These early approaches have largely given way to the more efficient phosphoramidite and H-phosphonate approaches to synthesis. Beaucage and Caruthers, *Tetrahedron Lett.* 22, 1859-1862 (1981), discloses the use of deoxynucleoside phosphoramidites in polynucleotide synthesis. Agrawal and Zamecnik, U.S. Patent No. 5,149,798 (1992), discloses optimized synthesis of oligonucleotides by the H-phosphonate approach.

Both of these modern approaches have been used to synthesize oligonucleotides having a variety of modified internucleotide linkages. Agrawal and Goodchild, *Tetrahedron Lett.* 28, 3539-3542 (1987), teaches synthesis of oligonucleotide methylphosphonates using phosphoramidite chemistry. Connolly et al., *Biochemistry* 23, 3443 (1984), discloses synthesis of oligonucleotide phosphorothioates using phosphoramidite chemistry. Jager et al., *Biochemistry* 27, 7237 (1988), discloses synthesis of oligonucleotide phosphoramidates using phosphoramidite chemistry. Agrawal

et al., *Proc. Natl. Acad. Sci. USA* 85, 7079-7083 (1988), discloses synthesis of oligonucleotide phosphoramidates and phosphorothioates using H-phosphonate chemistry.

Solid phase synthesis of oligonucleotides by the foregoing methods involves the same generalized protocol. Briefly, this approach comprises anchoring the 3'-most nucleoside to a solid support functionalized with amino and/or hydroxyl moieties and subsequently adding the additional nucleosides in stepwise fashion. Desired internucleoside linkages are formed between the 3' functional group of the incoming nucleoside and the 5' hydroxyl group of the 5'-most nucleoside of the nascent, support-bound oligonucleotide.

Oligonucleotide synthesis generally begins with coupling, or "loading," of the 3'-most nucleoside of the desired oligonucleotide to a functionalized solid phase support. A variety of solid supports and methods for their preparation are known in the art. *E.g.*, Pon, "Solid-Phase Supports for Oligonucleotide Synthesis," in *Methods in Molec. Biol. , Vol. 20,: Protocols for Oligonucleotides and Analogs*, p. 465 (Agrawal, Ed., Humana Press, 1993). Generally, the functionalized support has a plurality of long chain alkyl amines (LCAA) on the surface that serve as sites for nucleoside coupling. Controlled pore glass (CPG) is the most widely used support. It consists of approximately 100-200 μm beads with pores ranging from a few hundred to a few thousand angstroms.

Refinement of methodologies is still required, however, particularly when making a transition to large-scale synthesis (10 μmol to 1 mmol amd higher). Padmapriya et al., *Antisense Res. Dev.* 4, 185 (1994). Several modifications of the standard phosphoramidite

methods have already been reported to facilitate the synthesis (Padmapriya et al., *supra*; Ravikumar et al., *Tetrahedron* 50, 9255 (1994); Theisen et al., *Nucleosides & Nucleotides* 12, 1033 (1993); Bonora, *Nucl. Acids Res.* 21, 1213 (1993); Habus and Agrawal, *Nucl. Acids Res.* 22, 43 (1994); and Iyer et al., *Nucleosides & Nucleotides* 14, ** (1995) (in press)) and isolation (Kuijpers et *al. Nucl. Acids Res.* 18, 5197 (1990); and Reddy et al., *Tetrahedron Lett.* 35, 4311 (1994)) of oligonucleotides.

Although the phosphotriester method of oligonucleotide synthesis (*see, e.g.*, Koziolkiewicz and Wilk in *Methods in Molecular Biology, Vol. 20,* pp. 207-220, *supra*) once dominated oligodeoxynucleotide preparation, there have been no reports of the synthesis of oligonucleotides having multiple phosphotriester linkages. There is still an unfulfilled need for methods of producing such oligonucleotides and for the oligonucleotides themselves.

The routine synthesis of oligonucleotides is presently carried out using various N-acyl protecting groups for the nucleoside bases, such as isobutyryl (for guanine), and benzoyl for adenine and cytosine. After the synthesis of the oligonucleotides is carried out using either phosphoramidite chemistry or H-phosphonate chemistry, the protecting groups are removed by treatment with ammonia at 55-60°C for 5-10 hours. Using these protecting groups, PO oligonucleotides and other modified oligonucleotides can be synthesized. But in certain instances where modified oligonucletides are functionalized with base-sensitive groups, the functionalities often get removed while the deprotection is being carried out. Examples of such base-sensitive modified oligonucleotides include,

methyl phosphotriester oligonucletides, phosohsphoramides, etc. in other applications of oligonucletides, it is desirable to have oligonucleotides still bound to a solid-support. Such completely deprotected oligonucleotides still bound to the solid support will be useful in a variety of applications such as those involving isolation of transcription factors and other factors or elements that interact with oligonucleotides, solid-phase PCR, investigations into nucleic acid protein interactions by, for example, NMR, creation and use of combinatorial libraries, screening of nucleic acid libraries, and solid support based hybridization probes (analogous to Southern and Northern blotting protocols).

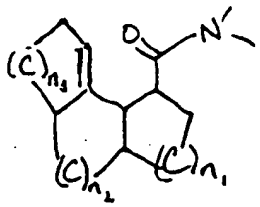
PS-oligos synthesized by prior art methods are mixtures of 2^n diastereomers, where n is the number of internucleotide phosphorothioates. To date only limited data is available on the comparative biophysical and biological properties of stereodefined phosphorothioates due to non-availability of sufficient quantities of completely "stereoregular" PS-oligos of sufficient length. Stec, *Angew-Chem. Int. Ed. Engl.* 33, 709 (1994) and references therein; Lesnikowski, J *Bioorg- Chem.*, 21, 127 (1993); Stec and Lesnikowski in *Methods in Molecular Biology, Vol. 20, supra*, pp. 285-313 and references cited therein; and Tang et al., *Nucleosides Nucleotides*, in press (1995). Enzymatic synthesis (Tang et al., *supra*) gives only R_p-phosphorothioates and is not as yet amenable to large-scale work.

The effects of different diastereomers on the efficacy of antisense oligonucleotides for gene modulation remain largely unknown. The potential impact of stereoselective synthesis to augment antisense oligonucleotide efficacy, however, is great. There is a

need, therefore, for further research to increase knowledge in this area and to develop methods for large-scale synthesis of "stereoregular" PS-oligos. Concomitantly, there is a need for additional synthetic research tools to aid in this endeavor.

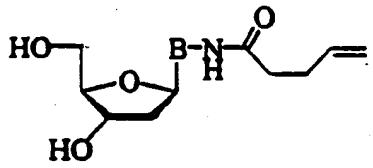
SUMMARY OF THE INVENTION

In one aspect of the present invention, a novel nucleoside base protecting group is provided. This protecting group has the general structure **XXI**:



where n_1, n_2, and n_3 are independently 0-10 and the nitrogen displayed is the amino moiety of the base.

In a preferred embodiment, compound **XXI** takes the form N-pent-4-enoyl $CH_2=CH(CH_2)_2CO$- (**XXII**). Compounds **XXI** and **XXII** protect the nucleoside base amino moieties by forming amide linkages, as in:



where the nitrogen is the amino moiety of the base B. Compound **XXI** and the preferred embodiment **XXII** are particularly advantageously used because they can be removed chemoselectively by treatment with a reagent such as I_2. The use of this mild procedure for removing the protecting group without affecting the integrity of other functionalities

present in the oligonucleotide makes it possible to prepare novel analogs of oligonucleotides such as alkyl phosphotriesters and other base-sensitive oligonucleotides. this new protecting group is compatible with H-phosphonate chemistry as well as phosphoramidite chemistry. Besides being able to synthesize oligonucleotides bearing "sensitive" functionalities, it can also be used in the routine synthesis of various oligonucleotides as in case of the conventional protecting groups. With incorporation of this protecting group into nucleoside bases, it is possible to synthesize oligonucleotides still bound to any type of solid support.

Accordingly, in another aspect of the invention, oligonucleotides having 3 or more phosphotriester internucleotide linkages are provided. These oligonucleotides are synthesized according to the method outlined in Figure 2.

In another aspect of the invention, phosphorothioate oligonucleotides bound or unbound to a solid support are provided. This aspect of the invention is is schematically displayed in Figure 3. If free (unbound) oligonucleotide is desired, the oligonucleotide can be synthesized according to the phosphoramidite method using the base protecting groups of the invention. When the full length support bound oligonucleotide is complete, it can be contacted with ammonia for 1 to 2 hours to yield the free, unprotected oligonucleotide.

Where an unprotected, support-bound oligonucleotide is desired, the full length support-bound oligonucleotide is contacted with I_2 in water to cleave the base protecting group and then with anhydrous triethylamine to cleave the β-cyano moiety.

Because the base-protecting group can also be used to protect hydroxyl moieties, support-bound branched oligonucleotides can be synthesized using, for example, glycol residues in which one hydroxyl group is protected by DMT and the other by a protecting group according to the invention. Then the DMT group may be selectively removed and an oligonucleotide synthesized from the resulting unprotected hydroxyl. Upon completion of that oligonucleotide, the hydroxyl moiety protected by the protecting group according to the invention can be deprotected with I_2 and water and another, different oligonucleotide synthesized from it. Such an approach is useful in producing combinatorial libraries.

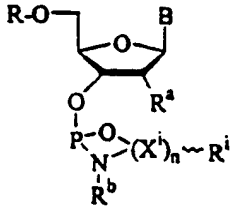
The unprotected support bound oligonucleotides of the invention can have phosphodiester, or phosphotriester internucleotide linkages of the form -O-PO(XR)-O- where X is O, NH, or S and R is a C_1 - C_{20} álkyl or an aryl moiety.

The resulting unprotected support-bound oligonucleotide is useful for a variety of purposes such as applications involving isolation of transcription factors and other factors or elements that interact with oligonucleotides, solid-phase PCR, investigations into nucleic acid protein interactions by, for example, NMR, creation and use of combinatorial libraries, screening of nucleic acid libraries, and solid support based hybridization probes (analogous to Southern and Northern blotting protocols).

The present invention also provides additional methods for the synthesis of oligonucleotides. These methods use phosphoramidite chemistry with novel phosphoramidite reagents, which form another aspect of the invention. The novel synthons and synthetic methods of the invention can be used for the synthesis of

diastereomeric mixtures of oligonucleotides and for the synthesis of oligonucleotides enriched in a particular diastereomer. The invention also provides oligonucleotides produced from these compounds by these methods. Oligonucleotides produced using the synthons and methods of the invention are useful for any purpose for which oligonucleotides produced using prior art techniques are used, such as PCR and as inhibitors of nucleic acid expression.

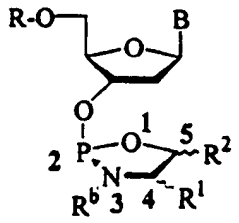
In one aspect of the invention, we provide monomer synthons having the structure:



XIII

where B is any suitably protected purine or pyrimidine base or derivative thereof and each of the chiral X^i have a well defined stereoconfiguration. According to the methods of the present invention, compounds of structure XIII can be made to be diastereomerically enriched or a mixture of diastereomers. Diastereomerically enriched synthons as well as derivatives and analogs thereof are useful in the synthesis of diastereomerically pure oligonucleotides by the phosphoramidite method. They can be used as a substitute for the well-known β-cyanoethyl-protected phosphoramidate.

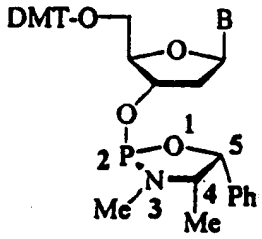
In a preferred embodiment, synthon XIII has the form:



XIV

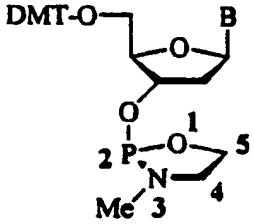
where chiral centers 4 and 5 can be, respectively, R and S, S and R, R and R, or S and S.

In a particularly preferred embodiment of this aspect of the invention, synthon XIII has the form:



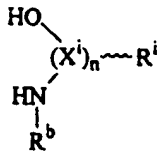
VI

In another particularly preferred embodiment, the present invention provides a phosphoramidite monomer synthon VIa:



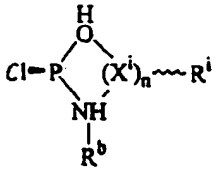
VIa

In another aspect of the invention, a method of synthesizing the diastereomerically enriched monomer synthons XIII, XIV, and VI is provided. The method comprises contacting



XVII

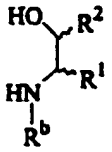
to yield



XVIII

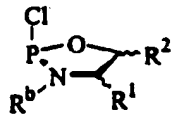
which, when contacted with a 5′-protected mononucleoside having an unprotected 3′ hydroxyl, yields **XIII**.

In a preferred embodiment of this aspect of the invention, **XVII** takes the form:



XIX

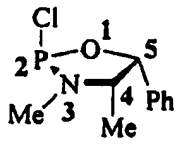
and yields



XX

which can be reacted with a mononucleoside to yield **XIV**.

In a particularly preferred embodiment of this aspect of the invention, (1R, 2S)-(-)-ephedrine (V) with PCl_3 to yield the chlorophosphoramidite product:

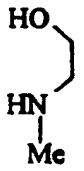


IV

The R_p isomer predominates (>95%). Contacting IV with a 5´-DMT-protected mononucleoside having a free 3´ hydroxyl group yields the monomer synthon VI in high yield (84%).

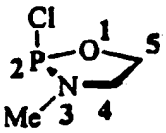
Each of the foregoing reactions is stereoretentive. Thus, particular diastereomers of each of XIII, XIV, and VI can be obtained by starting with the appropriate stereoisomer of XVII, XIX, and V, respectively.

In another particularly preferred embodiment of the invention, the monomer synthon VIa is synthesized by contacting:



XIXa

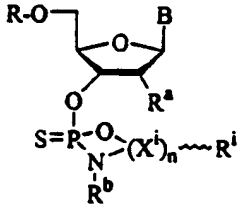
with PCl_3 to yield



IVa

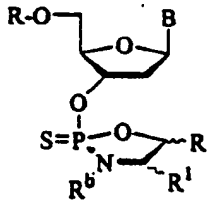
which can then be contacted with a 5′-protected mononucleoside having an unprotected 3′-hydroxyl to yield VIa.

In another aspect of the invention, thiophosphoramidate monomer synthons enriched in a particular stereoisomer are provided. These compounds have the general structures:



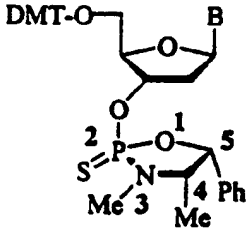
XV

and



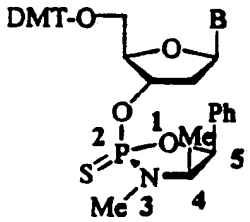
XVI

In a preferred embodiment of this aspect of the invention, thiophosphoramidate monomer synthons having *syn*-(VIIIb) and *anti*- (VIIIa) conformations are provided:



anti-isomer

VIIIa

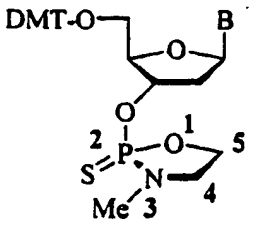


syn-isomer

VIIIb

XV, XVI, VIIIa and VIIIb are all made by oxidatively thiolating the monomer synthon precursors XIII, XIV, and VI, respectively, with a sulfurizing agent such as the 3H-1,2-benzodithiole-3-one-1,1-dioxide reagent. The result is about 90% retention of configuration. The stereoisomers can be separated by flash chromatography.

In a particularly preferred embodiment of this aspect of the invention, the phosphorothioate monomer synthon VIIIc is provided:



VIIIc

VIIIc is made by oxidatively thiolating the monomer synthon precursor VIa with a sulfurizing agent such as the 3H-1,2-benzodithiole-3-one-1,1-dioxide reagent.

In another aspect of the invention, oligonucleotides having one or more P-chiral centers predominantly in the S configuration and methods for their synthesis have been developed. In one embodiment of this aspect of the present invention, these oligonucleotides can be synthesized via the well-known phosphoramidate approach using **XIII**, **XIV**, or **VI** instead of the well known β-cyanoethyl phosphoramidite synthon. The intermediate phosphite linkage may be oxidized with, for example, I_2 and H_2O in THF to yield a phosphodiester linkage, or oxidatively thiolated with a sulfurizing agent, such as the Beaucage reagent, to yield a phosphorothioate linkage. Oligonucleotides synthesized according to this embodiment of the invention will have predominantly S_p configuration (~60%) at each internucleotide linkage in which compound **XIII**, **XIV**, or **VI** was employed during synthesis.

In another embodiment of this aspect of the invention, one of **XV**, **XVI**, **VIIIa** or **VIIIb** is contacted with a nascent oligonucleotide having a free 5′ hydroxyl group. When either **VIIIa** or **VIIIb** is used, the result is an oligonucleotide having a 5′

phosph rothioate internucleotide linkage with an R_p:S_p ratio of about 70:30 when VIIIa is used and 10:90 when VIIIb is used. Similar results are obtained from compound XVI when R^1 and R^2 are both *anti-* or both *syn-* with respect to the nucleoside and compound XV when all of the R^i are *anti-* or *syn-* with respect to the nucleoside.

As noted, each of the foregoing monomer synthons and oligonucleotides can be synthesized using the methods of the present invention to be in enantiomeric excess. One advantage of the methods of the present invention is that the stereochemistry of the precursors is maintained in the products, and, if the reactants are in enantiomeric excess, the products are predominantly in one stereoconfiguration.

When stereochemistry is unimportant, however, the present method provides a convenient method of oligonucleotide synthesis using the phosphoramidite method wherein the well-known β-cyanomethol phosphoramidite synthon is replaced by compound VIa. The intermediate phosphite linkage may be oxidized with, for example, I_2 and H_2O in THF to yield a phosphodiester linkage, or oxidatively thiolated with a sulfurizing agent, such as the Beaucage reagent, to yield a phosphorothioate linkage.

In an alternative embodiment, compound VIIIc is used to synthesize oligonucleotides having phosphorothioate internucleotide linkages by contacting VIIIc with a nascent oligonucleotide having a free 5′-hydroxyl group.

Oligonucleotides according to the invention are useful for both *in vitro* and *in vivo* applications. For *in vitro* applications, the present oligonucleotides are useful as research

tools in determining gene function by effecting gene modulation and as hybridization probes, for example.

Oligonucleotides according to the invention are also useful for *in vivo* applications, such as the treatment of pathogen-caused diseases. Oligonucleotides according to the invention can be synthesized to have a sequence sufficiently complementary to a region of a nucleic acid essential for the growth, reproduction, and/or metabolism of the pathogen to inhibit expression of the nucleic acid under physiological conditions.

The foregoing merely summarizes certain aspects of the present invention and is not meant, nor should it be construed, to limit the invention in any way. All patents and other publications cited herein establish the state of the art and are hereby incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

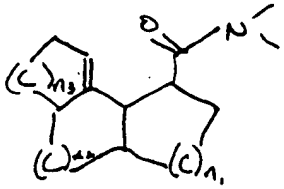
Figure 1 displays the synthetic pathway for making the compounds of the present invention.

Figure 2 displays synthesis of phosphodiester and phosphotriester oligonucleotides using H-phosphonate chemistry and the base protecting group of the invention.

Figure 3 displays synthesis of free and support-bound unprotected oligonucleotides according to the method and reagents of the invention.

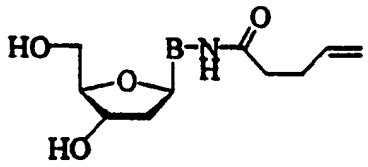
DETAILED DESCRIPTION OF THE PREFERRED EMBODIMIENTS

In one aspect of the present invention, a novel nucleoside base protecting group is provided. This protecting group has the general structure **XXI**:



where n_1, n_2, and n_3 are independently 0-10 and the nitrogen displayed is the amino moiety of the base.

In a preferred embodiment, compound **XXI** takes the form N-pent-4-enoyl $CH_2=CH(CH_2)_2CO$- (**XXII**). Compounds **XXI** and **XXII** protect the nucleoside base amino moieties by forming amide linkages, as in:



where the nitrogen is the amino moiety of the base B. Compound **XXI** and the preferred embodiment **XXII** are particularly advantageously used because they can be removed chemoselectively by treatment with a reagent such as I_2. The use of this mild procedure for removing the protecting group without affecting the integrity of other functionalities present in the oligonucleotide makes it possible to prepare novel analogs of oligonucleotides usch as alkyl phosphotriesters and other base-sensitive oligonucleotides. this new protecting group is compatible with H-phosphonate chemistry as well as

phosphoramidite chemistry. Besides being able to synthesize oligonucleotides bearing "sensitive" functionalities, it can also be used in the routine synthesis of various oligonucleotides as in case of the conventional protecting groups. With incorporation of this protecting group into nucleoside bases, it is possible to synthesize oligonucleotides still bound to any type of solid support.

Accordingly, in another aspect of the invention, oligonucleotides having 3 or more phosphotriester internucleotide linkages are provided. These oligonucleotides are synthesized according to the method outlined in Figure 2. After the complete support-bound oligonucleotide is synthesized according to the H-phosphonate method using the base amino-protecting group of the present invention, the oligonucleotide is contacted carbon tetrachloride, N-methyl imidazole and RXH (where X is O, NH, or S and R is a C_1-C_{20} alkyl or aryl) and then with iodine in water to remove the protecting group.

In another aspect of the invention, phosphorothioate oligonucleotides bound or unbound to a solid support are provided. This aspect of the invention is is schematically displayed in Figure 3. If free (unbound) oligonucleotide is desired, the oligonucleotide can be synthesized according to the phosphoramidite method using the base protecting groups of the invention. When the full length support bound oligonucleotide is complete, it can be contacted with ammonia for 1 to 2 hours to yield the free, unprotected oligonucleotide.

Where an unprotected, support-bound oligonucleotide is desired, the full length support-bound oligonucleotide is contacted with I_2 in water to cleave the base protecting group and then with anhydrous triethylamine to cleave the β-cyano moiety.

Because the base-protecting group can also be used to protect hydroxyl moieties. In this aspect of the invention, support-bound branched oligonucleotides can be synthesized using, for example glycol residues in which one hydroxyl group is protected by DMT and the other by a protecting group according to the invention. Then the DMT group may be selectively removed and an oligonucleotide synthesized from the resulting unprotected hydroxyl. Upon completion of that oligonucleotide, the hydroxyl moiety protected by the protecting group according to the invention can be deprotected with I_2 and water and another, different oligonucleotide synthesized from it.

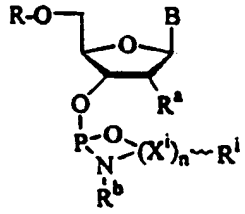
The unprotected support bound oligonucleotides of the invention can have phosphodiester, or phosphotriester internucleotide linkages of the form -O-PO(XR)-O- where X is O, NH, or S and R is a C_1 - C_{20} alkyl or an aryl moiety.

The resulting unprotected support-bound oligonucleotide is useful for a variety of purposes such as applications involving isolation of transcription factors and other factors or elements that interact with oligonucleotides, solid-phase PCR, investigations into nucleic acid protein interactions by, for example, NMR, creation and use of combinatorial libraries, screeing of nucleic acid libraries, and solid support based hybridization probes (analogous to Southern and Northern blotting protocols).

The present invention also provides a sructurally novel class of antisense oligonucleotides useful for modulation of nucleic acid expression *in vitro* and *in vivo*. The present invention also provides novel methods for synthesizing this class of oligonucleotides using new synthons.

The present invention also provides additional methods for the synthesis of oligonucleotides. These methods use phosphoramidite chemistry with novel phosphoramidite reagents, which form another aspect of the invention. The novel synthons and synthetic methods of the invention can be used for the synthesis of diastereomeric mixtures of oligonucleotides and for the synthesis of oligonucleotides enriched in a particular diastereomer. The invention also provides oligonucleotides produced from these compounds by these methods. Oligonucleotides produced using the synthons and methods of the invention are useful for any purpose for which oligonucleotides produced using prior art techniques are used, such as PCR and as inhibitors of nucleic acid expression.

In one aspect of the invention, we provide monomer synthons having the structure:



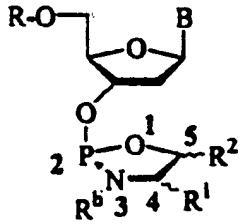
XIII

wherein R^a and R^b, and each R^i are independently H, C_1 - C_{20} alkyl, aryl, heterocyclic, C_1 - C_{20} alkoxy, R is a suitable protecting group, such as DMT, n is 1-3, i is 1-n, X^i is C, O,

S, or N, such that if n > 1 the identity of each X^i (*i.e.*, each of $X^1 \ldots X^n$) is independent of the identity of every other X^i and the identity of each substituent R^i (*i.e.*, $R^1 \ldots R^n$) is independent of every other R^i, each R^i is covalently bound to the corresponding X^i (*e.g.*, X^1-$R^1 \ldots X^n$-R^n), the X^i are arranged consecutively such that X^1 is bound to the N and X^n is bound to the O, and B is any suitably protected, modified or unmodified, purine or pyrimidine base. As used herein, the term "aryl" means a polyaromatic-ring structure having from 1 to 5 linearly or angularly fused aromatic rings, such as phenyl and naphthyl. As used herein the term "heterocyclic" means a 5 or 6 membered ring having from 1 to 5 heteroatoms (*i.e.*, N, S, or O) that may be located at any position within the ring. Furan and thiophene are examples of heterocyclic moieties encompassed by this definition. Compound XIII is synthesized according to the methods of the present invention (*infra*) to be predominantly in one stereoconfiguration.

The stereochemistry of the product XIII depends on the stereochemistry of the starting material. Synthesis of XIII from its precursor is accomplished in a stereorentive manner, *infra*.

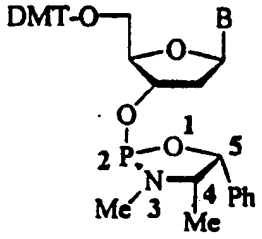
In a preferred embodiment of this aspect of the invention, R^a is H, n is 2, and X^1 and X^2 are each C, which has the structure XIV:



XIV

In this embodiment, the configurations at carbons 4 and 5 can be, respectively, R and S, S and R, R and R, or S and S, each of which can be obtained in pure form.

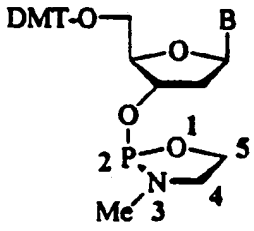
In a particularly preferred embodiment of this aspect of the present invention, n is 2, X^1 and X^2 are each C, R^1 is methyl, R^2 is phenyl, R^a is H, R^b is methyl, and the compound has the R_p configuration as shown in structure VI:



VI

All of the four diastereomers of VI (in which carbons 4 and 5 are in the (R,S), (S,R), (R,R), and (S,S) configurations) can be made from one of the stereoisomers of precursor IV, *infra*.

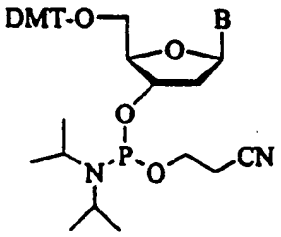
In another particularly preferred embodiment, the present invention provides a phosphoramidite monomer synthon VIa:



VIa

In addition, the present method provides a method of synthesizing oligonucleotides according to the phosphoramidite method using the phosphoramidite VIa. The method comprises the synthon VIa with a nascent oligonucleotide having a free 5'-hydroxyl group. The chemistry is otherwise the same as standard phosphoramidite chemistry.

Myriad suitable base protecting groups are known to those skilled in the art. *E.g.*, Sonveaux in *Methods in Molecular Biology, v. 26: Protocols for Oligonucleotide Conjugates* pp. 1-72 (S. Agrawal, Ed., Humana Press (1994)) and references cited therein. Similarly, numerous modified bases are known to those skilled in the art. *E.g.*, Meyer, *Methods in Molecular Biology, v. 26, supra*, pp. 73-92 and references cited therein. Synthons XIII, XIV, and VI and derivatives thereof are useful in the synthesis of oligonucleotides by the phosphoramidite method, as discussed more fully below. It can be used as a substitute for the well-known β-cyanoethyl-protected phosphoramidate:

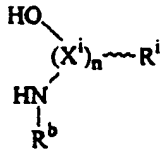


XII

Furthermore, the synthetic protocol for incorporating XIII, XIV, and VI in a nascent oligonucleotide is the same as that for the β-cyanoethyl-protected phosphoramidates. *E.g.*, Beaucage in *Methods in Molecular Biology, Vol. 20, Protocols for Oligonculeotides and Analogs, supra*, pp. 33-61 and references cited therein. For simplicity, as used herein

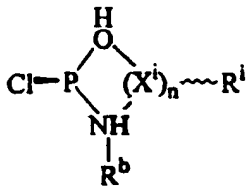
the term "nascent oligonucleotide" means a solid support-bound nucleotide chain having at least one nucleotide.

In another aspect of the invention, a method of synthesizing the diastereomerically enriched monomer synthon **XIII** is provided. In this aspect of the invention PCl_3 is reacted with a compound of structure **XVII**:



XVII

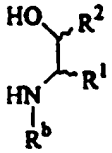
to yield



XVIII

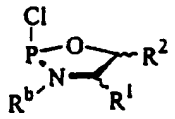
where each of X^i, R^i, R^b, and n are defined the same as described for compound **XIII**. **XVIII**is contacted with a 5'-protected mononucleoside having an unprotected 3'-hydroxyl to yield **XIII**. Compounds **XIII** and **XVIII** are obtained from their precursors (**XVIII** and **XVII**, respectively) a stereoretentive manner, *i.e.*, the stereoconfiguration of the precursor is maintained in the reaction.

Compound **XIV** is obtained in a similar manner by contacting **XIX**



XIX

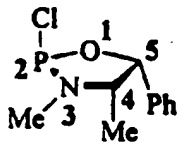
with PCl_3 to yield



XX

wherein each of R^1 and R^2 are the same as R^i in compound XIII and R^b is the same as in compound XIII. Compound XIV is obtained by contacting XX with a 5'-protected mononucleoside having an unprotected 3'-hydroxyl. As before, the reactions are stereoretentive, and the products of each reaction, XIV and XX, retain the same stereoconfiguration as their precursor, XX and XIX, respectively.

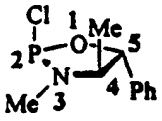
In a preferred embodiment of this aspect of the invention, a method of synthesizing the diastereomerically enriched monomer synthon VI is provided. The method comprises contacting (1R, 2S)-(-)-ephedrine (V) with PCl_3 at between -100 and 40 °C for between one and 40 hours. In a preferred embodiment, the two compounds are allowed to react in N-methyl morpholine and toluene at -78 °C for 3 hours and then at 22 °C for 12 hours. Other suitable solvents are benzene, tetrahydrofuran, ether, and dioxane. The result is about a 75% yield of the chlorophosphoramidite product:

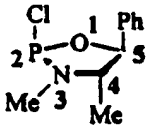


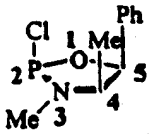
IV

The R_p isomer predominates (>95%). Contacting IV with a 5′-DMT-protected m nonucle side having a free 3′-hydroxyl group yields the monomer synthon VI in high yield (84%). In a preferred embodiment, the mononucleoside and IV are allowed to react in ethyl ether and triethylamine as a scavenger of HCl liberated during the reaction. Other scavengers such as pyridine and 2,6-lutidine can also be used. The reaction can be conducted at temperatures ranging from between -100 and 40 °C for between 1 and 40 hours. In a preferred embodiment, the mixture is allowed to react at -78 °C for 3 hours and then at 22 °C for 12 hours. Other suitable solvents such as benzene, tetrahydrofuran, ether, and dioxane can also be used. Compound IV is fairly stable, undergoing no apparent decomposition (as evaluated by ^{31}P-NMR) after being stored at -5 °C for several days.

The other stereoisomers of ephedrine (1S,2R; 1S,2S; and 1R,2R) (V) are also commercially available and can be used in place of (1R,2S)-(-)-ephedrine (V) to obtain the other diastereomers of IV:



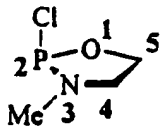




In another particularly preferred embodiment of the invention, the monomer synthon VIa is synthesized by contacting:

XIXa

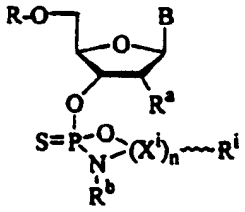
with PCl_3 to yield



IVa

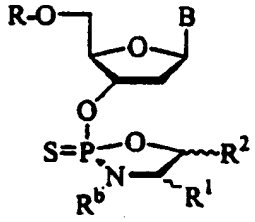
which can then be contacted with a 5'-protected mononucleoside having an unprotected 3'-hydroxyl to yield VIa. The same reaction conditions as described previously can be used.

In another aspect of the invention, thiophosphoramidate monomer synthons enriched in a particular stereoisomer are provided. These compounds have the general structures:



XV

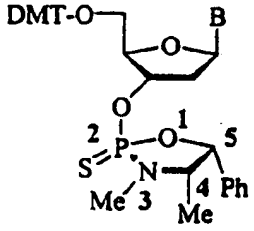
and



XVI

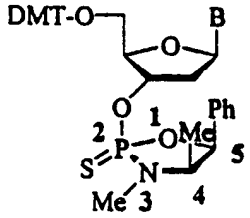
wherein the definitions of B, R, R^i, R^a, R^b, R^1, R^2, X^i, i, and n are the same as described previously for compound XIII. Monomer synthons XV and XVI are obtained in the predominant configurational stereoisomer by stereoretentive oxidative thiolation of the phosphorous of compounds XIII and XIV, respectively. Oxidative thiolation of a particular stereoisomer of XIII or XIV (provided above) results in approximately 90% conversion to the corresponding thiophosphoramidate stereoisomer.

In a preferred embodiment of this aspect of the invention, the *anti-* isomer of synthon VIII is provided. The *syn-* (VIIIb) and *anti-* (VIIIa) forms of the synthon VIII according to this aspect of the invention have the following structures:



anti-isomer

VIIIa

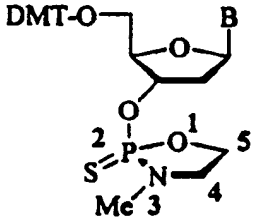


syn-isomer

VIIIb

VIIIa and VIIIb are made by oxidatively thiolating the monomer synthon VI. The result is a 90:10 *anti:syn* mixture.

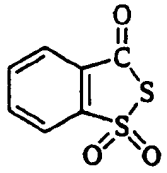
In a particularly preferred embodiment of this aspect of the invention, the phosphorothioate monomer synthon VIIIc is provided:



VIIIc

VIIIc is made by oxidatively thiolating the monomer synthon precursor VIa

Any suitable method of oxidative thiolation may be used, such as elemental sulfur. *E.g.*, Stec et al., *J Am. Chem. Soc.* **106**, 6077 (1984). Preferably, the thiophosphoramidate monomer synthons are synthesized by contacting the phosphoramidite precursors with the Beaucage reagent, 3H-1,2-benzodithiol-3-one-1,1-dioxide:



I

according to the method of Iyer et al., *J. Am. Chem. Soc.* **112**, 1253 (1990) and Iyer et al., *J. Org Chem.* **55**, 4693 (1990). In a preferred embodiment, reagent I is used as a 2% solution in acetonitrile and the mixture is allowed to react for 30 seconds at about room temperature. All of the various diastereomers (*e.g.*, VIIIa and VIIIb) are easily separated by conventional chromatography or crystallization.

In another aspect of the invention, oligonucleotides having from one to all nucleotide P-chiral centers independently predominantly in the S configuration and methods for synthesizing them are provided. As used herein, the term "predominantly" means more than half. In one embodiment of this aspect of the present invention, these oligonucleotides can be synthesized via the well-known phosphoramidate approach (*e.g.*, Beaucage in *Methods in Molecular Biology, Vol 20, Protocols for Oligonculeotides and Analogs, supra*, pp. 33-61 and references cited therein) using XIII in place of XII. In a

preferred embodiment of this aspect of the invention, XIV is used in place of XII. In a particularly preferred embodiment, VI is used.

In brief, a nascent oligonucleotide having a free 5′ hydroxyl is contacted with XIV, XV, or VI in the presence of tetrazole. A phosphate linkage is thereby formed. The phosphite linkage may then be oxidized with, for example, I_2 and H_2O in THF to yield a phosphodiester linkage or oxidatively thiolated with I to yield a phosphorothioate linkage. Phosphorothioate oligonucleotides synthesized according to this embodiment of the invention have predominantly S_p configuration (~60%) at each internucleotide linkage in which compound XIV, XV, or VI was employed during synthesis.

When stereochemistry is unimportant, however, the present method provides a convenient method of oligonucleotide synthesis using the foregoing synthetic methods with compound VIa. In an alternative embodiment, compound VIIIc is used to synthesize oligonucleotides having phosphorothioate internucleotide linkages by contacting VIIIc with a nascent oligonucleotide having a freee 5′-hydroxyl group.

In another embodiment of this aspect of the invention, oligonucleotides having one or more phosphorothioate internucleotide linkages that are independently predominantly in the R or S configuration are provided. In this embodiment, one of the stereoisomers of XV or XVI is contacted with a nascent oligonucleotide having an unprotected 5′ hydroxyl group. In a preferred embodiment, VIIIa or VIIIb is used, resulting in an oligonucleotide having a 5′ phosphorothioate internucleotide linkage with an R_p:S_p ratio of about 70:30 (starting with VIIIa) or 10:90 (starting with VIIIb). Similar results are obtained from

compound XVI when R^1 and R^2 are both *anti-* or both *syn-* with respect to the nucleoside and compound XV when all of the R^1 are *anti-* or *syn-* with respect to the nucleoside.

The antisense oligonucleotides of the present invention may be designed to incorporate a number of additional features that have been demonstrated to increase efficacy. For example, they may be designed to be "self-stabilized," *i.e.*, having a first region sufficiently complementary to a second region to allow for intramolecular hybridization, thereby rendering the oligonucleotide less susceptible to nucleolytic attack. Such oligonucleotides are described in PCT International Application Publication No. WO 94/01550.

All of the foregoing methods can be used with RNA and DNA and with any solid support. *See, e.g.,* Pon in *Methods in Molecular Biology, Vol. 20*, pp. 465-496.

Alternatively, the presently disclosed oligonucleotides may be designed to be "fold-back triplex forming," *i.e.*, having a first region complementary to a target nucleic acid and a second region having a sequence that allows for triplex formation by Hoogsteen base pairing between it and the duplex formed by the first region and the target nucleic acid, as described in PCT International Application Publication No. WO 94/17091.

Oligonucleotides according to the invention are useful for both *in vitro* and *in vivo* applications. For *in vitro* applications, the present oligonucleotides are useful as research tools in determining gene function. Because they can be prepared to be complementary to a particular sequence, the present oligonucleotides can be used to selectively inhibit expression of a target gene. The present oligonucleotides thus provide an attractive and

easily used alternative to the laborious method of gene inhibition by mutation (*e.g.*, deletion mutation). The significance of this will be appreciated when one realizes that the elucidation of most biological pathways now known has been determined by deletion mutations.

Oligonucleotides according to the invention are also useful in standard hybridization assays.

The oligonucleotides of the present invention are also useful as therapeutic agents for diseases or physiological conditions involving expression of specific genes. Oligonucleotides useful for treating a disease or condition will have a nucleotide sequence sufficiently complementary to the target nucleic acid to bind to the target nucleic acid under physiological conditions. As used herein, the terms "complementary" and "sufficiently complementary" are used interchangeably and, when used to describe the sequence of an antisense oligonucleotide, mean that the oligonucleotide sequence is such that the oligonucleotide inhibits expression of the target nucleic acid under the conditions of interest (*e.g.*, *in vitro* experimental conditions or physiological conditions). In general, oligonucleotides according to the invention will have a sequence complementary to a nucleic acid (*e.g.*, a gene or mRNA) that is essential to a biological process. As elaborated more fully below, such processes include reproduction and metabolic processes of pathogens and other disease-causing infectious agents. Alternatively, the biological process can be a naturally occurring one whose inhibition is desirable, *e.g.*, spermatogenesis in men and ovulation in women desiring contraception. The

oligonucleotides of the invention can also be complementary to a gene or other nucleic acid whose expression causes or is involved in a diseased or otherwise abnormal state of the organism.

Because of their efficacy at gene modulation, the presently claimed oligonucleotides are also useful for treating diseases arising from genetic abnormalities that cause under- or over-expression of a gene. For diseases in which an abnormal gene is expressed or a normal gene is over-expressed, for example, the presently claimed oligonucleotides may be designed to target the abnormal or normal gene directly, or, in the alternative, to target the gene encoding the protein that promotes expression of the abnormal or normal gene. Conversely, where a normal gene is under-expressed, one may design an oligonucleotide that suppresses expression of a gene encoding a protein that suppresses expression of the normal gene.

In many cases the target nucleic acid sequence will be a viral nucleic acid sequence. The use of antisense oligonucleotides to inhibit various viruses is well known and has been reviewed in Agrawal, *Trends in Biotechnology 10*, 152 (1992). Viral nucleic acid sequences that hybridize to effective antisense oligonucleotides have been described for many viruses, including human immunodeficiency virus type I (U.S. Patent No. 4,806,463), Herpes simplex virus (U.S. patent No. 4,689,320), Influenzavirus (U.S. Patent No. 5,194,428), and Human papilloma virus (Storey et al., *Nucleic Acids Res.* 12, 4109 (1991)). Sequences hybridizing to any of these nucleic acid sequences can be used, as can nucleotide sequences complementary to nucleic acid sequences from any other

virus. Additional viruses that have known nucleic acid sequences against which an antisense oligonucleotide according to the invention can be prepared include, but are not limited to, Foot and Mouth Disease Virus (See Robertson et al., *J. Virology* 54, 651 (1985); Harris et al., *J. Virology* 36, 659 (1980)), Yellow Fever Virus (*see* Rice et al., *Science* 229, 726 (1985)), Varicella-Zoster Virus (*see* Davison and Scott, *J. Gen. Virology* 67, 2279 (1986), Cucumber Mosaic Virus (*see* Richards *et al.*, *Virology* 89, 395 (1978)), Hepatitis B Virus (*see* Raney and McLachlen, in *Molecular Biology of Hepatitis B Virus* (CRC Press, 1991)), Hepatitis C Virus (*see* Miller and Purcell, *Proc. Natl. Acad. Sci. USA* 87, 2057 (1990); *Proc. Natl Acad. Sci. USA* 89, 4942 (1992); *J. General Virology* 74, 661 (1993)), and Respitory Syncitial Virus (*see* Collins, in *The Paramyxo Viruses*, Chapter 4, pp. 103-162 (David W. Kingsbury, Ed., 1991)).

Alternatively, the oligonucleotides of the invention can have a nucleotide sequence complementary to a nucleic acid sequence of a pathogenic organism. The nucleic acid sequences of many pathogenic organisms have been described, including the malaria organism, *Plasmodium falciparum* and many pathogenic bacteria. Examples of pathogenic eukaryotes having known nucleic acid sequences against which oligonucleotides of the present can be prepared include, but are not limited to *Trypanosoma brucei gambiense and Leishmania* (*see* Campbell et al., *Nature* 311, 350 (1984)), and *Fasciola hepatica* (*see* Zurita et al., *Proc. Natl. Acad Sci. USA* 84, 2340 (1987)). Antifungal oligonucleotides can be prepared having a nucleotide sequence that is complementary to a nucleic acid

sequence from, *e.g.*, the chitin synthetase gene, and antibacterial oligonucleotides according to the invention can be prepared using, *e.g.*, the alanine racemase gene.

In yet another embodiment, the oligonucleotides can have a nucleotide sequence complementary to a cellular gene or gene transcript, the abnormal expression or product of which results in a disease state. The nucleic acid sequences of several such cellular genes have been described, including prion protein (Stahl and Prusiner, *FASEB J.* 5, 2799 (1991)), the amyloid-like protein associated with Alzheimer's disease (PCT International Application Publication No. WO 95/09236), and various well-known oncogenes and proto-oncogenes, such as *c-myb, c-myc, c-abl, and* n-*ras*..

In addition, oligonucleotides that inhibit the synthesis of structural proteins or enzymes involved largely or exclusively in spermatogenesis, sperm motility, the binding of the sperm to the egg or any other step affecting sperm viability may be used as contraceptives for men. Similarly, contraceptives for women may be oligonucleotides that inhibit production of proteins or enzymes involved in ovulation, fertilization, implantation or in the biosynthesis of hormones involved in those processes. Hypertension can be controlled by oligonucleotides that suppress the synthesis of angiotensin converting enzyme or related enzymes in the renin/angiotensin system; platelet aggregation can be controlled by suppression of the synthesis of enzymes necessary for the synthesis of thromboxane A2 for use in myocardial and cerebral circulatory disorders, infarcts, arteriosclerosis, embolism and thrombosis; deposition of cholesterol in arterial wall can be inhibited by suppression of the synthesis of fatty acyl co-enzyme A: cholesterol acyl

transferase in arteriosclerosis; inhibition of the synthesis of cholinephosphotransferase may be useful in hypolipidemia.

There are numerous neural disorders in which oligonucleotides of the present invention can be used to reduce or eliminate adverse effects of the disorder. For example, suppression of the synthesis of monoamine oxidase can be used in Parkinson's disease; suppression of catechol O-methyl transferase can be used to treat depression; and suppression of indole N-methyl transferase can be used in treating schizophrenia.

Suppression of selected enzymes in the arachidonic acid cascade (which leads to prostaglandins and leukotrienes) may be useful in the control of platelet aggregation, allergy, inflammation, pain and asthma. Suppression of the protein expressed by the multi-drug resistance (mdr) gene, which is responsible for development of resistance to a variety of anti-cancer drugs and is a major impediment in chemotherapy may prove to be beneficial in the treatment of cancer. Nucleotide sequences complementary to nucleic acid sequences from any of these genes can be used for the oligonucleotides according to the invention, as can be oligonucleotide sequences complementary to any other cellular gene or gene transcript, the abnormal expression or product of which results in a disease state.

Antisense regulation of gene expression in plant cells has been described in U.S. Patent No. 5,107,065, and the antisense oligonucleotides of the invention can potentially be applied in this context as well.

Since the nucleotide sequence of the oligonucleotide can be adapted to form Watson-Crick base pairs with essentially any gene, the therapeutic spectrum of the oligonucleotides of the invention should be very broad. Still, certain diseases are of particular interest. For example, a variety of viral diseases may be treated by oligonucleotides having one or more S-triesterphosphorothioates internucleotide linkages, including AIDS, ARC, oral or genital herpes, papilloma warts, flu, foot and mouth disease, yellow fever, chicken pox, shingles, HTLV-leukemia, and hepatitis. Among fungal diseases treatable by oligonucleotides according to the invention are candidiasis, histoplasmosis, cryptococcocis, blastomycosis, aspergillosis, sporotrichosis, chromomycosis, dematophytosis and coccidioidomycosis. The method can also be used to treat rickettsial diseases (*e.g.*, typhus, Rocky Mountain spotted fever), as well as sexually transmitted diseases caused by *Chlamydia trachomatis* or *Lymphogranuloma venereum.*

A variety of parasitic diseases can be treated by oligonucleotides of the present invention, including amebiasis, Chagas' disease, toxoplasmosis, pneumocystosis, giardiasis, cryptosporidiosis, trichomoniasis, and *Pneumocystis carini* pneumonia; also worm (helminthic diseases) such as ascariasis, filariasis, trichinosis, schistosomiasis and nematode or cestode infections. Malaria can be treated by oligonucleotides of the present invention, regardless of whether it is caused by *P. falciparum, P.vivax, P. orale,* or *P. malaria.* The infectious diseases identified above can all be treated with oligonucleotides according to the invention because the infectious agents and their gene sequences f r these

diseases are known, and, thus, oligonucleotides according to the invention can be prepared having a nucleotide sequence that hybridizes to a nucleic acid sequence that is an essential nucleic acid sequence for the propagation of the infectious agent, such as an essential gene. As used herein, an essential gene or nucleic acid is one that is required for a biological process and without which the biological process does not occur.

The following examples are provided for illustrative purposes only and are not intended, nor should they be construed, as limiting the invention in any way.

EXAMPLES

Unless otherwise stated, all chemicals recited in the following Examples were obtained from Aldrich of Milwaukee, WI.

Example 1

Stereoselective Synthesis of a Mononucleotide Synthon

The chlorophosphoramidite, (2R,4S,5R)-2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine (IV) was obtained by mixing 8.14 g of 1R,2S-ephedrine (V) and 10.4 ml of N-methyl morpholine in 250 ml of toluene under argon and cooling to -78 °C. 4.3 ml of PCl_3 in 10 ml of toluene was added over a period of 15 minutes. The mixture was kept at -78 °C for 1 hour and then allowed to warm to room temperature over a period of 16 hours. the insoluble salt precipitate was filtered under argon. The precipitate was washed with 3 x 25 ml of toluene. The combined washings and filtrate were concentrated *in vacuo* in a rotary evaporator to remove toluene. Vacuum distillation of the residue gave a colorless liqued boiling at 0.1 mm Hg at 95 °C to give ca. 9 g (80% yield) of the product. This procedure is similar to that described previously. Sun et al., *J. Chem. Soc. Perkin Trans. I* p. 3183 (1994) and references therein and Carey et al., *J. Chem. Soc. Perkin Trans. I,* p. 831 (1993).

^{31}P-NMR examination of the resulting crude reaction mixture revealed the presence of- a predominant isomer (> 95%) at δ 169.4 ppm and a minor component (< 5%) at δ 161 ppm. Upon vacuum distillation of the reaction mixture (95-97 °C at 0.1 mm Hg), a col rless liquid was btained, which solidified to a white crystalline mass upon cooling

to -78 °C (isolated yields of 75%). Carey et al., *supra*, reported a b.p. of 160 °C at 0.1 mm Hg. NMR analysis gave the following results: ^{31}P-NMR ($CDCl_3$) (TMP external standard) δ 169.1 ppm; ^{1}H-NMR ($CDCl_3$) δ (ppm) 0.71 (3H, d, J=6.3 Hz), 2.69 (3H, d, $^3J_{P-H}$ = 15.1 Hz, N-CH_3), 3.63 (1H, ddq, J=1.3, 5.5, $^3J_{P-H}$=7.6 Hz, H-4), 5.85 (1H, dd, j = 5.5 Hz, $^3J_{P-H}$ ~ 1.2 Hz), 7.15 (5H, m, -Ph). These spectral features are in agreement with values reported by Sun et al. and Carey et al., *supra*, and lead to the assignment of structure IV as being the R isomer in which the chlorine atom is disposed *trans* relative to the C-Ph and C-Me substituents in the phospholidine ring. IV could be stored as a solid in a desiccator at -5 °C for several days with no apparent decomposition (as evaluated by ^{31}P-NMR). Upon addition of water to IV, the H-phosphonate VII was obtained as a mixture of disstereomers (R_p:S_p, 55:45 ^{31}P-NMR).

2.16 g of 5'-*O*-dimethoxytrityl thymidine was dissolved in a mixture of anhydrous ether (20 ml) and anhydrous triethylamine (5 ml). The solution was added gradually (10 min) to 1.2 g of the chlorophosphoamidite (IV) at room temperature and the solution stirred at room temperature for 6 hours. The reation mixture was poured into 200 ml of ice-cold water. It was then extracted with ethylacetate (3 x 200 ml). The combined organic layer was washed with water. The organic layer was evaporated to dryness to give 3 g (84 % yield) of VI as a white foamy material.

Synthesis of XIII and XIV is conducted according to the same protocol.

The ^{31}P-NMR spectrum of VI has a signal at δ 140 ppm, corresponding to a single P-epimer. In analogy with substitution reactions of VI involving carbon-, oxygen-, and

nitrogen-based nucleophiles (Sun et al. and Carey et al., *supra*), which gave substitution products with overall retention of configuration, VI can be formulated as having the structure with R_p configuration (Fig.1). This hitherto unreported nucleoside phosphoramidite VI is a white solid and is stable when stored dry at 0 - 5 °C. The NMR and mass spectral features of VI are as follows: ^{31}P-NMR ($CDCl_3$) (TMP ext. standard) δ 169 ppm; ^{1}H-NMR ($CDCl_3$) δ (ppm) 0.61 (3H, d, J = 6.5 Hz), 1.41 (3H, s, T-CH), 2.42 (2H, m, H-2′), 2.63 (3H, d, $^3J_{P-H}$ = 12 Hz, N-CH_3), 3.37 (1H, dd, J = 10.6, 2.6 Hz, H-5′), 3.46 (1H, dd, J = 10.6, 2.6 Hz, H-5′), 3.52 (1H, ddq, J = 6.9, 6.5 Hz, $^3J_{P-H}$ = 2.4 Hz, H-4), 3.76 (6H, s, -OCH_3), 4.08 (1H, m, H-4′), 4.91 (1H, m, H-3′), 5.56 (1H, dd, J = 6.9 Hz, $^3J_{P-H(5)}$ = 1.84 Hz, H-5), 6.41(1H, dd, J = 6.7, 6.7 Hz, H-1′), 6.85 (4H, m, -Ph), 7.25 (14H, m, -Ph), -7.6 (1H, s, H-6), 9.1 (1H, s, -NH). FAB-MS (m/z) = 736 (M-H), C_4,$H_{44}N_3O_8P$.

Oxidative sulfurization of the phosphoramidite VI with thiolsulfonate I (R.I. Chemicals, Costa Mesa, CA) according to Iyer *et al.*, *J. Am. Chem. Soc.* **112**, 1253 (1990), and Iyer et al., *J. Org. Chem.* **55**, 4693 (1990) gave the thiophosphoramidates **VIIIa: VIIIb** (90:10, 81 % yield) (isomer ratio based on ^{31}P-NMR. The NMR and mass spectral features were as follows: **VIIIa**, ^{31}P-NMR ($CDCl_3$) δ (ppm) 79.0; ^{1}H -NMR ($CDCl_3$) δ (ppm) 0.78 (3H, d, J = 6.6 Hz, -CH) 1.41 (3H, s, T-CH3) 2.55 (2H, m, H-2′), 2.70 (3H, d, $^3J_{P-H}$ = 12.5 Hz, -NCH_3), 3.36 (1H, dd, J = 10.5, 2.3 Hz, H-5′), 3.56 (1H, dd, J = 10.5, 2.2 Hz, H-5′) 3.76 (1H, ddq, J = 6.6, 6.1 $^3J_{P-H}$ = 12.3 Hz, H-4), 3.78 (6H, s, -OCH_3), 4.28 (1H, m, H-4′), 5.57 (1H, m, H-3′), 5.62 (1H, dd, J = 6.1 Hz,

$^3J_{P\text{-}H(5)}$ = 2.8 Hz, H-5), 6.48 (1H, dd, J = 9.0, 5.6 Hz, H-1´), 6.85 (4H, m, -Ph), 7.26 (14H, m, -Ph), 7.62 (1H, s, H-6) 8.90 (1H, s, -NH). FAB-MS (m/z) 769, $C_{41}H_{44}N_3O_8PS$.

The predominant isomer, **VIIIA** (which is easily separated from **VIIIb** by flash chromatography), has been assigned the configuration indicated in Fig. 1. The assignment of configurations for **VIIIa** and **VIIIb** is based on the generally accepted notion that P(III) oxidations proceed with high stereoselectivity and with overall retention of configuration. *E.g.*, Beaucage and Iyer, *Tetrahedron* **48**, 2223 (1992), and Bentrude et al., *J. Am. Chem. Soc.* **111**, 3981 (1989).

Example 2

Synthesis of Nucleotide Dimers Using Diastereomerically Enriched Monomer Synthons

Having obtained the nucleoside phosphoramidite **VI** in preparative-scale reactions, the stage was set for its use in solid-phase coupling with CPG-bound nucleoside. Thus, contacting a solution of **VI** in acetonitrile with CPG-T (10 mmol) for a period of two minutes in the presence of tetrazole as an activator followed by oxidation with the thiolsulfonate **I** resulted in efficient formation of the product **IX** with a coupling efficiency of greater than 95% (as evaluated by "trityl yields"). Iyer et al., *J. Am. Chem. Soc., supra,* and Iyer et al., *J Org. Chem., supra.* Following synthesis, the CPG-bound product was heated with aqueous ammonium hydroxide (28%, 55 °C, 1 hr). Examination of the products by reverse-phase HPLC revealed that the dinucleoside phosphorothioate **X** had been formed as a mixture of diastereomers (R_p:S_p, 40:60). Interestingly, the commonly used cyan ethylphosphate deprotecti n strategy (28% aq. NH_4OH, 55 °C) was found to

be sufficient to cleave the chiral phosphate appendage in IX and generate the phosphorothioate X. The lack of high stereoselectivity in the formation of X is consistent with other reports wherein epimerization of the phosphorous center (in the case of stereoisomerically pure phosphoramidites) is observed when acidic type activators, *e.g.*, tetrazole, are used in conjunction with phosphoramidite methodology in the synthesis of deoxyribonucleoside phosphorothioates. Stec, *supra*, and Beaucage, *supra*.

Example 3

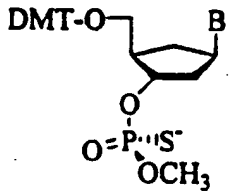
Synthesis and Purification of Oligonucleotides

Oligonucleotides are synthesized on a 1 mmol scale following the standard protocol by using an automated synthesizer (*e.g.*, Millipore 8700 DNA Synthesizer, Bedford, MA). Where a predominantly R_p configuration is desired, the phosphoramidite VI is used by dissolving it in dry acetonitrile at a concentration of 50 mg/ml. For phosphorothioate oligonucleotides, the iodine oxidation step is replaced by sulftirization with 3H-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent). Iyer *et al.*, *J. Org. Chem.* **55**, 4693 (1990). Two-hour treatment with ammoniun hydroxide at room temperature is carried out to cleave the oligomer from the support and to deprotect nucleoside bases. Oligonucleotides are purified by reverse-phase HPLC and/or PAGE, and desalted by using C-1 SEP-PAK cartridges.

Example 4

Stereoselective Synthesis of a Mononucleotide Phosphorothioate

Treatment of **VIIIa** and **VIIIb** with sodium methoxide in methanol at ambient temperature overnight followed by heating with NH_4OH (28% NH_4OH for 1-2 hr at 55 °C gave the phosphorothioate:



XI

in 90% yield with moderate to high stereoselectivity (as monitored by ^{31}P-NMR and HPLC). The R_p:S_p ratio of **XI** obtained from **VIIIa** was 70:30, whereas the ratio of isomers obtained from **VIIIb** was 10:90. Configurations were assigned using the criteria reported for dinucleoside phosphorothioates by Iyer et al., *Bioorg. Med. Chem. Lett.* **4**, 2471 (1994).

Example 5

Stereospecific Phosphorothioate Synthesis

Diazabicyclononane (DBU) (296 mg, 1.95 mmol) is dissolved in anhydrous THF (1.5 ml) and added to 3'-*O*-t-butyl dimethylsilyl thymidine (46 mg, 0.129 mmol) at 0 °C for 20 minutes. This solution is added slowly to the solution of **VIIIa** (50 mg, 0.065 mmol) and the contents stirred for 30 minutes at room temperature. The reaction mixture is allowed to warm to room temperature and stirred for 12 h. The s lution is evap rated

to remove solvent and treated with ammonium hydroxide (28%, 1 ml) and heated for 4 h at 55 °C. The solution is evaporated to dryness. Chromatographic purification affords 45 mg (70 % yield) of 5'-*O*-DMT-3'-*O*-TBDMS TT dimer with R_p:S_p ratio of 70:30.

Example 6

Cellular Uptake

Cell culture

Human T cell and leukemia cell line H9 are used in this study. They are cultured in RPMI media supplemented with 10% fetal bovine serum (heat inactivated to 56 °C for 30 minutes to inactivate the nucleases), 2 mM glutamine, 100 ml streptomycin, 100 U/ml penicillin and 6 x 10^{-5} M of 2-mercaptoethanol in an air incubator (37 °C, humidified by 5% CO_2-95% O_2).

Fluorescein labeling of oligonucleotides

Fluorescein is conjugated to the 5´ end of the oligonucleotides by either an automated DNA synthesizer or by a manual procedure using a "FLUORESCEIN-ON" phosphoramidite. The efficiency of fluorescein labeling is determined by using a spectrofluorometer (excitation 488 nm, emission 520 nm).

Cell uptake

The concentrations of the fluorescein labeled and unlabelled oligonucleotides in the samples are measured by a spectrofluorometer and UV spectroscopy and adjusted to be the same by adding the corresponding unlabelled oligonucleotides. Labelled oligonucleotides (0.2 OD/100 ml) are added to the cells (5 x 10^6 cells/ml, 0.5 ml) and set to culture. After

4 hours of culture, aliquots of cell culture mixtures are removed, washed, and resuspended in Hank's balanced salt solution (HBSS) supplemented with 0.1 % BSA and 0.1 % sodiun azide. Propidium iodide (final concentration 10 μl/ml) is used to distinguish viable cells from dead cells. Flow cytometric data on 5,000 viable cell is acquired in list mode on Epics XL (Coulter, Hialeah, FL), and data are analyzed by Epics XL (version 1.5 software) after gating on living cells by forward scatter versus side scatter and propidium iodide staining.

The results demonstrate that oligonucleotides according to the invention are taken up by cells.

Example 7

Inhibition of HIV-1 Replication

The following assays are used to measure the ability of the oligonucleotide of the invention to inhibit HIV-1 replication.

Syncytia Assay

The ability of the oligonucleotides of the invention to inhibit HIV-1 replication, and thus syncytia formation, in tissue culture is tested in T cell cultures according to the method of Agrawal and Sarin, *Advanced Drug Delivery Rev.* 6, 251 (1991). Briefly, CEM cells are infected with HIV-1 virions (0.01 - 0.1 $TCID_{50}$/cell) for one hour at 37°C. After one hour unadsorbed virions are washed and the infected cells are divided among walls of 24 well plates. To the infected cells, an appr priate concentrati n (fr m stock solution)

of oligonucleotide is added to obtain the required concentration in 2 ml medium. The cells are then cultured for three days. At the end of three days, infected cells are examined visually for syncytium formation or stained with trypan blue or CTT for cytopathic effect determination.

The results demonstrate that oligonucleotides according to the invention inhibit syncitia formation.

p24 Expression Assay

HIV expression can be determined by measuring the level of viral protein p24 expression in CEM cells essentially as described by Agrawal and Sarin, *supra*. Briefly, cells are pelleted and then resuspended in phosphate saline at a concentration of about 10^6/ml. The cells are spotted on toxoplasmosis slides, air dried, and fixed in methanol/acetone (1:1) for 15 min at room temperature (RT). The slides are next incubated with 10% normal goat serum at RT for 30 min and washed with phosphate buffered saline (PBS). Anti-p24 monoclonal antibody is added to each well, and the slides are incubated in a humid chamber at 37°C. The slides are labelled with goat anti-mouse IgG for 30 min and then washed in PBS overnight. The percentage of cells fluorescing in oligonucleotide-treated and untreated cells is compared.

The results demonstrate that oligonucleotides according to the invention substantially and significantly reduce p24 expression.

Cytopathic Effect (CPE)

HIV-induced cytopathic effect is determined by measuring the decrease in the number of viable cells after infection. The cells are counted by adding MTT or trypan blue dye to the cells and determining how many cells (dead) take up the dye. The assay is done in triplicate.

The results demonstrate that oligonucleotides according to the invention will reduce the viral cytopathic effect.

Reverse Transcriptase Assay

This assay is performed essentially as described in Agrawal and Sarin, *supra*. Supernatants from virus-infected cultures in the presence and absence of oligonucleotide are collected and virus particles precipitated with poly(ethyleneglycol). The virus pellet is suspended in 300 μl of buffer containing 50 mM Tris-HCl (pH 6.8), 5 mM dithiothreitol (DTT), 250 mM KCl, and 25% Triton X-100. Reverse transcriptase activity in the solubilized pellet is assayed in a 50 μl reaction mixture containing 50 mM Tris-HCl (pH 7.8), 5 mM DTT, 100 mM KCl, 0.01% Triton X-100, 5 μg dt15.rAn as template primer, 10 mM MgCl2, 15 μM [^{3}H]dTTP (15 Ci/mmol), and 10 μl of the disrupted virus suspension. After incubation for 1 hr at 37°C and subsequent addition of 50 μg yeast tRNA, the incorporation into the cold trichloroacetic acid-insoluble DNA fraction is assayed by counting in a β scintillation counter.

The results demonstrate that oligonucleotides according to the invention inhibit reverse transcriptase.

Example 8

4-Pentenoic anhydride required for the preparation of the protected nucleoside is synthesized by a modification of the literature report. Ellervik, U and Magnusson, G. Acta Chemica Scandinavica, 1993, 47:826-828 as given below:

4-Pentenoic acid (25 g, 250 mmol) is dissolved in 200 ml of dichloromethane and the solution cooled in ice-bath. Triethyl amine (35 ml, 250 mmol) is added slowly to the cooled solution. Next, N,N-bis[oxo-3-oxazolidinyl]phosphorodiamidic chloride (prepared by the literature procedure, J. Cabre-Castellvi et al., Synthesis, 616-620, 1981) was added to the above reaction mixture. The reaction mixture is allowed to warm to room temperature and stirred for 2-3 h. The reaction slurry was filtered and the residue was washed with dichloromethane (ca. 100 ml). The combined filtrate and washings were combined and evaporated to give a pale yellow liquid. Vacuum distillation at 90-93°C/6 mm Hg gave 4-pentenoic anhydride as a colorless liquid ca. 20 g (85% yield).

Example 9

Preparation of N-pent-4-enoyl 2'-deoxy adenosine (dA Npr):

2'-Deoxy adenosine (Mallinkckrodt) (2.5 g, 10 mmol) is dried by repeated evaporation from anhydrous pyridine and is suspended in 50 ml of anhydrous pyridine. Trichloromethylsilane (64. ml, 50 mmol) is added and the reaction is stirred for about 1 h. Then, 4-pentenoic anhydride (4g, 20 mmol) is added and the contents stirred. After 15 min triethyl amine (3 ml) was added and the contents stirred for 2-3 h. The reaction slurry was cooled to 0-5°C and 10 ml of water was added. After 5 min., 28% NH4OH (10

ml) was added. The resulting clear solution was evaporated to dryness. Water (150 ml) was added and the reaction mixture was extracted with ethylacetate:ether (50 ml, 1:1). The aqueous layer was separated and concentrated to a small volume. Upon leaving at room temperature, a white precipitate of the title compound was obtained. Filtration and drying gave ca. 3.5 g of pure title compound. Several experiments repeating the above procedure, using larger scale of operation, gave the title compound in 85-90% yield.

Same general procedure can be employed for the preparation of dG and dC protected nucleosides.

Example 10

Preparation of 5'-O-DMT-N-4-pent-4-enoyl-2'-deoxyadenosine-3'-H-phosphonate (triethyl ammonium salt)

The title compound was prepared by adopting a procedure as described by Froehler in Protocols for Oligonucleotides and analogs, Agrawal, S. Ed., pp. 63-80 as given below:

To 544 mg (1.63 mmol) of dA(N-pr) in 20 ml of anhydrous pyridine is added 1.108 g (3.3 mmol) of dimethoxytritylchloride. The reaction mixture is stirred at room temperature for 12 h. The reaction mixture is evaporated to dryness. The residue is chromatographed over silica gel 60 and eluted with CH_2Cl2:CH_3OH:(Et)3N to give 0.73 of 5'-O-DMT-N-4-pent-4-enoyl-2'-deoxyadenosine as a white foamy material.

To a stirred solution of 1,2,4 triazole (0.944 g, 13.3 mmol) and triethyl amine (5.5 ml, 30 mmol) in anhydrous CH_2Cl_2 (40 ml) is added PCl_3 (0.35 ml, 3.9 mmol) at room temperature under argon. After 30 min, the reacti n mixture is cooled to 0°C and 5'-

DMT-protected nucleoside (500 mg, 0.88 mmol) in 15 ml CH_2Cl_2 is added dropwise over 10-15 min at 0°C and allowed to warm to room temperature. The reaction mixture is poured into 1M triethylammoniumbicarbonate (TEAB) (75 ml, pH 8.5) with stirring. The mixture is transferred to a separatory funnel and the phases are separated. The aqueous phase is extracted with methylene chloride and the combined organic phase washed with 1M TEAB (1 x 50 ml). The organic layer is dired over sodium sulfate and evaporated to dryness. The solid product thus obtained is purified by chromatography over silica gel. Elution with CH_2Cl_2:CH_3OH:$(Et)_3N$ (18:1:1) gave 0.065 g of the title compound.

Other H-phosphonate nucleosides were similarly prepared in overall yields ranging from 70-90%.

Similarly nucleoside 5'-O-DMt-3'-6-cyanoethyl-N,N-diisopropylphosphoramidites and 5'-O-DMT-3')-methyl-N,N-diisopropylphosphoramidites were prepared using standard protocols as described by Beaucage, S.L., in Protocols for Oligonucleotides and Analogs, Agrawal, S. Ed., pp. 33-61.

Example 11

Protocol for removal of the protecting group after dinucleoside synthesis (Eg. Tg-dimer):

The support bound synthesized by H-phosphonate methodology (Froehler ref. above) is treated with a solution of 2% I_2 in (pyridine:water, 98:2) for 30 min. This procedure completely removes the base protecting groups. This additional step is not necessary if one is making Po oligos using H-phosphonate methodology because

simultaneous oxidation and deprotection can be achieved by a single I_2 protocol using the reagent specified above.

Example 12

Protocol for preparation of phosphate methylated oligonucleotide [eg., TG (P-OMe)]:

The support-bound H-phosphonate oligonucleotide is treated with a 10% solution of MeOH (ROH) in CCl_4/N-methylimidiazole/Et_3N, 9/0.5/0.5 for 40 min. The solid support is washed with acetonitrile dried and then treated with the iodine solution as above to remove the base-protecting group. The CPG-bound oligonucleotide is next treated with a solution of K_2CO_3 in methanol (0.05 M) for 4-6 h. to cleave the oligo from the support. The solution is evaporated to dryness and the oligonucleotide can be purified by reverse phase HPLC to give the pure phosphatemethylated dimer.

Similar protocol can be employed for longer oligos except that the time of deprotection and oxidation have to be increased.

Other backbone modified oligonucleotides can be prepared by using ROH (for alkyl phosphotriesters), RNH_2 (for phosphoramidates).

Example 13

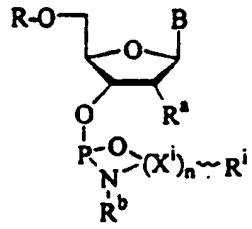
Protocol for preparation of phosphorothioates (free and support-bound):

Following the preparation of phosphorothioate oligonucleotide by phosphoramidite methodology, but using the new nucleobase protecting group, the support-bound oligo is treated with the iodine solution, as above, to remove the base-protecting group and then

with anhydrous triethylamine to remove the phosphate protecting group. Finally cleavage from the support is achieved by treatment with K_2CO_3 solution as above.

We claim:

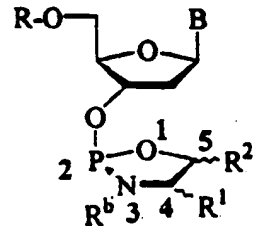
1. A mononucleotide synthon having the structure:



XIII

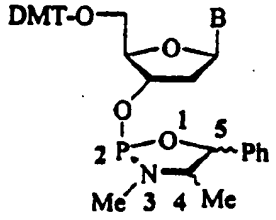
wherein R^a and R^b, and each R^i are independently H, C_1 - C_{20} alkyl, aryl, heterocyclic, C_1 - C_{20} alkoxy, R is a protecting group, n is 1-3, i is 1-n, X^i is C, O, S, or N, such that if n > 1 the identity of each X^i is independent of the identity of every other X^i and the identity of each substituent R^i is independent of every other R^i; each R^i is covalently bound to the corresponding X^i, the X^i are arranged consecutively such that X^1 is bound to the N and X^n is bound to the O, and B is any protected, modified or unmodified, purine or pyrimidine base.

2. A mononucleotide synthon according to claim 1 having the structure:

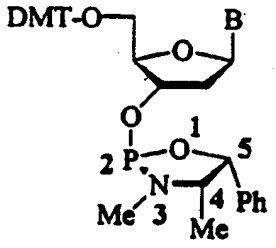


XIV

3. A mononucleotide synthon according to claim 2 having the structure:

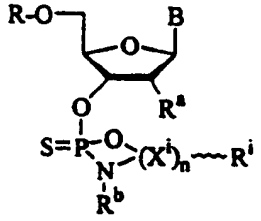


4. A mononucleotide synthon according to claim 3 having the structure and stereoconfiguration:



VI

5. A mononucleotide synthon having the structure:

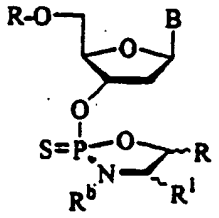


XV

wherein R^a and R^b, and each R^i are independently H, C_1 - C_{20} alkyl, aryl, heterocyclic, C_1 - C_{20} alkoxy, R is a protecting group, n is 1-3, i is 1-n, X^i is C, O, S, or N, such that if n > 1 the identity of each X^i is independent of the identity of every other X^i and the identity of each substituent R^i is independent of every other R^i; each R^i is covalently bound to the

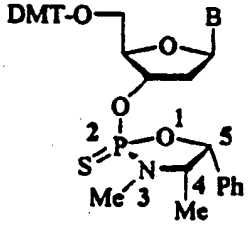
corresponding X^i, the X^i are arranged consecutively such that X^1 is bound to the N and X^a is bound to the O, and B is any protected, modified or unmodified, purine or pyrimidine base.

6. A mononucleotide synthon according to claim 5 having the structure:



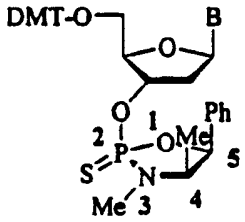
XVI

7. A mononucleotide synthon according to claim 6 having the structure and configuration:



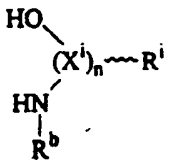
VIIIa

8. A mononucleotide synthon according to claim 6 having the structure and configuration:



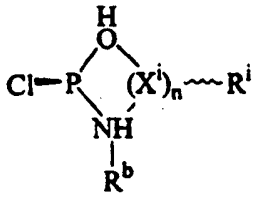
VIIIb

9. A method of synthesizing the mononucleotide synthon according to claim 1 comprising contacting a compound of structure:



XVII

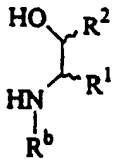
with PCl_3 to yield



XVIII

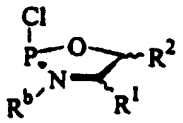
and then contacting XVIII with a 5′-protected monnucleoside having an unprotected 3′-hydroxyl.

10. A method according of synthesizing a mononucleotide synthon according to claim 2 comprising contacting a compound having structure:



XIX

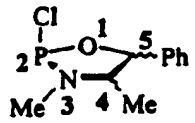
with PCl_3 to yield



XX

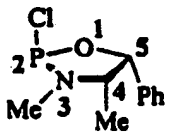
and then contacting XX with a 5′-protected monnucleoside having an unprotected 3′-hydroxyl.

11. A method of synthesizing the compound according to claim 3 comprising contacting ephedrine with PCl_3 to yield the chlorophosphoramidite:



and then contacting the chlorophosphoramidite with a 5′-protected mononucleoside having an unprotected 3′-hydroxyl.

12. A method of synthesizing the compound according to claim 4 comprising contacting (1R,2S)-(-)-ephedrine with PCl_3 to yield the chlorophosphoramidite:



IV

and then contacting IV with a 5'-protected mononucleoside having an unprotected 3'-hydroxyl.

13. A method of synthesizing the compound according to claim 5 comprising contacting compound XIII with oxidative thiolation agent.

14. A method according to claim 13, wherein the oxidative thiolation agent is 3H-1,2-benzodithiol-3-one-1,1-dioxide.

15. A method of synthesizing the compound according to claim 6 comprising contacting the compound XIV with oxidative thiolation agent.

16. A method according to claim 15, wherein the oxidative thiolation agent is 3H-1,2-benzodithiol-3-one-1,1-dioxide.

17. A method of synthesizing the compound according to claim 7 comprising contacting compound VI oxidative thiolation agent.

18. A method according to claim 17, wherein the oxidative thiolation agent is 3H-1,2-benzodithiol-3-one-1,1-dioxide.

19. An oligonucleotide having from one to all nucleotide P-chiral centers independently predominantly in the S configuration.

20. An oligonucleotide having from one to all phosphorothioate internucleotide linkages that are independently predominantly in the R or S configuration.

21. A method of synthesizing an oligonucleotide according to claim 19 comprising contacting a nascent oligonucleotide having a free 5'hydroxyl with compound XIV.

22. A method of synthesizing an oligonucleotide according to claim 19 comprising contacting a nascent oligonucleotide having a free 5'hydroxyl with compound XV.

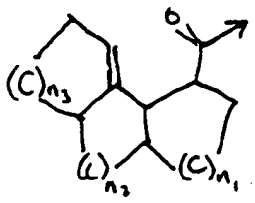
23. A method of synthesizing an oligonucleotide according to claim 19 comprising contacting a nascent oligonucleotide having a free 5'hydroxyl with compound VI.

24. A method of synthesizing an oligonucleotide according to claim 20 comprising contacting a nascent oligonucleotide having a free 5'hydroxyl with compound XV.

25. A method of synthesizing an oligonucleotide according to claim 19 comprising contacting a nascent oligonucleotide having a free 5'hydroxyl with compound XVI.

26. A method of synthesizing an oligonucleotide according to claim 19 comprising contacting a nascent oligonucleotide having a free 5'hydroxyl with compound VIIIa or VIIIb.

27. A nucleoside base amino protecting group having the structure:



where n_1, n_2, and n_3 are independently 0-10, and the arrow indicates the point of linkage to the amino moiety.

28. The nucleoside base amino protecting group according to claim 27 having the structure:

and the arrow indicates the point of linkage to the amino moiety.

29. An oligonucleotide comprising from 3 to all internucleoside linkages being phosphotriester linkages of the form:

where X is O, N, or S and R is a C_1 - C_{20} alkyl group or an aryl group.

30. A method of synthesizing a phosphotriester oligonucleotide comprising synthesizing a support bound oligonucleotide according to the H-phosphonate method using the base protecting according to either of claims 27 or 28, then contacting the support bound

31. A method of sythesizing an unprotected support bound oligonucleotide comprising synthesizing a support bound oligonucleotide according to the phosphoramidite method using the base protecting according to either of claims 27 or 28, contacting the support bound oligonucleotide with iodine in water, and then contacting the support bound oligonucleotide with anhydrous triethylamine.

32. A method of screening for compounds that preferentially bind to oligonucleotides having a particular sequence comprising contacting an composition containing the compound that preferentially binds to oligonucleotides having a particular sequence with a support bound oligonucleotide according to claim 31, wherein the support-bound oligonucleotide has the sequence to which the compound preferentially binds.

33. The method of claim 32 wherein the compound is a nucleic acid, a protein, or a transcription factor.

34. An unprotected solid support-bound oligonucleotide.

35. A combinatorial library comprising the oligonucleotide according to claim 34.

36. A method of screening compounds that preferentially bind to oligonucleotides having a particular sequence comprising contacting the compounds with a combinatorial library according to claim 35.

37. A mononucleotide synthon according to claim 2, wherein R^b is methyl and R^1 and R^2 are both H.

38. A mononucleotide synthon according to claim 6, wherein R^b is methyl and R^1 and R^2 are both H.

39. A method according to claim 10, wherein R^b is methyl and R^1 and R^2 are both H.

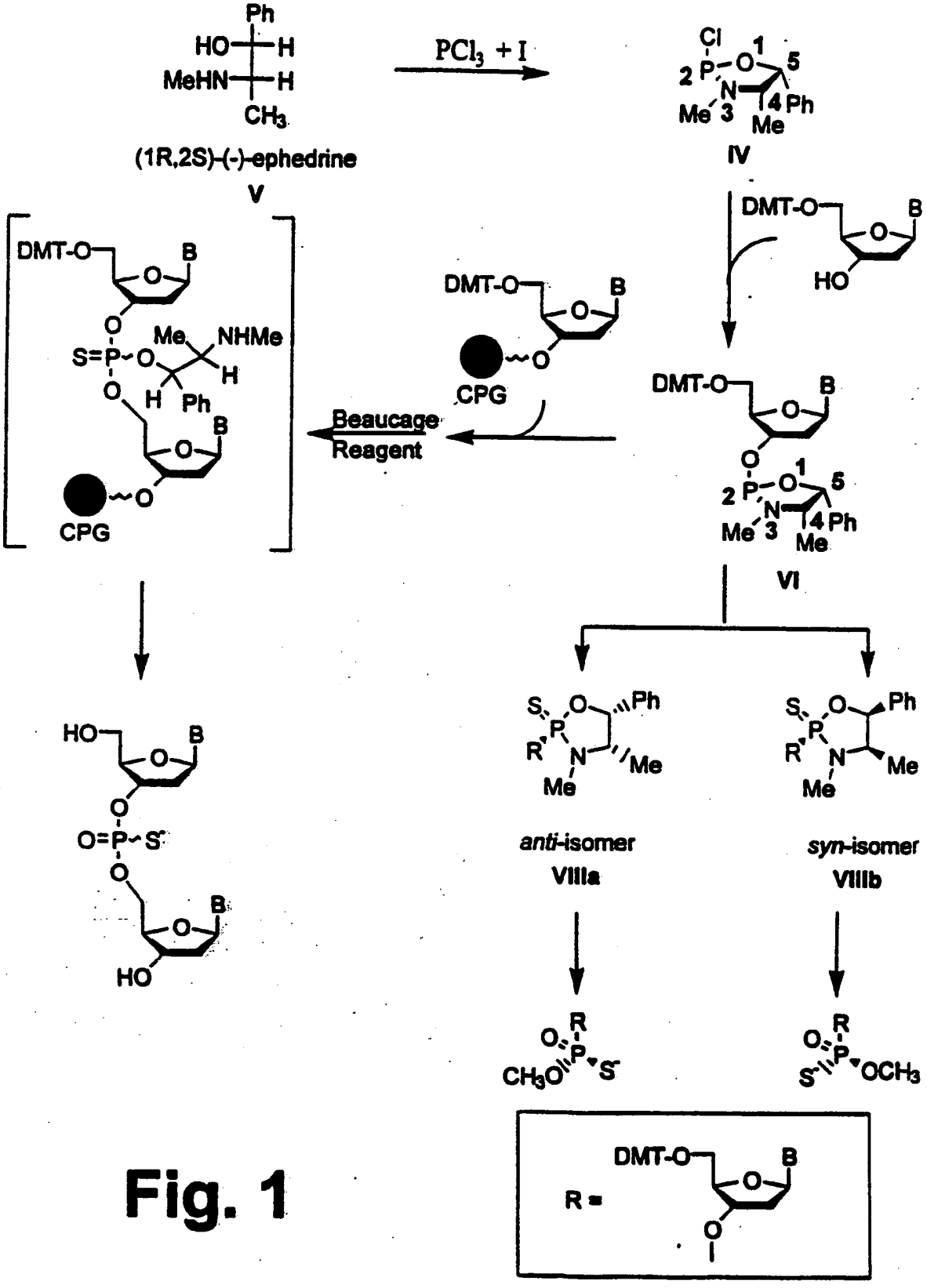

Ph
HO
H
MeHN
H
CH3
(1R,2S)-(-)-ephedrine
V
PCl3 + I
Cl
1
2
P
O
5
N
3
4 Ph
Me
Me
IV
DMT-O
B
O
HO
DMT-O
B
O
CPG
Beaucage
Reagent
DMT-O
B
O
Me
NHMe
S=P
O
H
H
Ph
O
B
O
CPG
DMT-O
B
O
2
P
O
1
5
N
3
4 Ph
Me
Me
VI
HO
B
O
O=P
S
O
B
O
HO
S
P
O
Ph
R
N
Me
Me
anti-isomer
VIIIa
S
P
O
Ph
R
N
Me
Me
syn-isomer
VIIIb
O
R
P
CH3O
S
O
R
P
S
OCH3
R =
DMT-O
B
O
O

Fig. 1

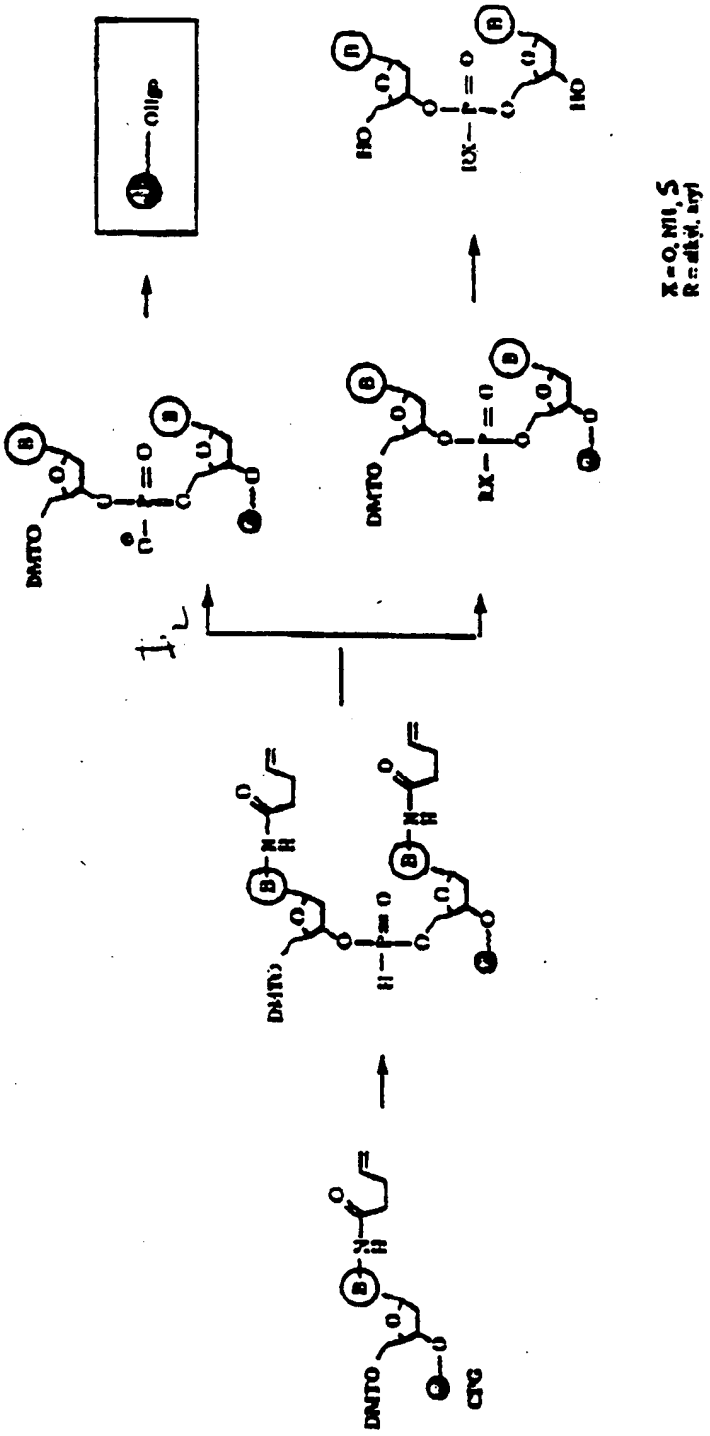


Fig. 2

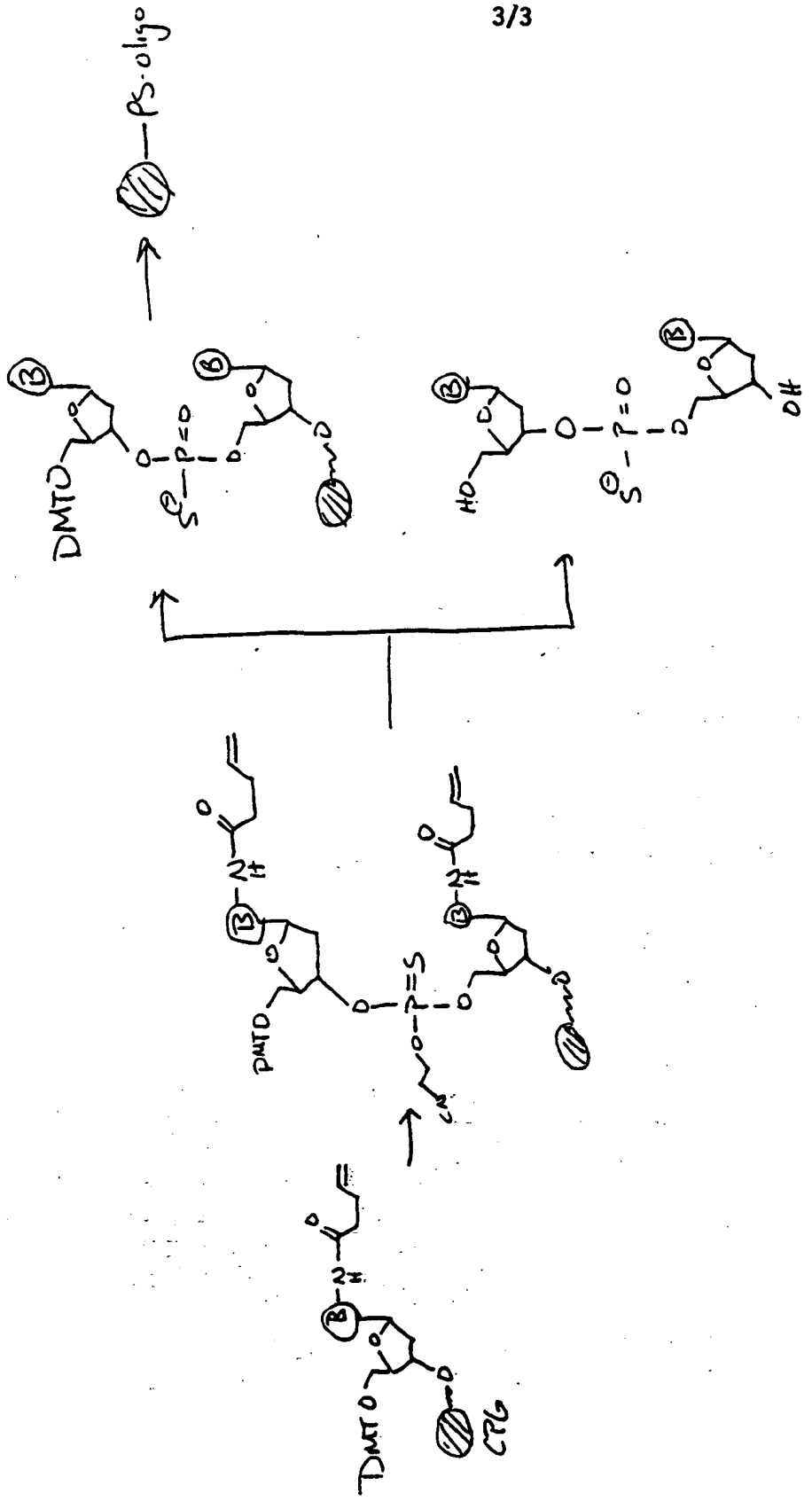


Fig. 3

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/07430

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H19/10 C07H21/00 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TETRAHEDRON: ASYMMETRY, vol. 6, no. 5, 26 May 1995, OXFORD GB, pages 1051-1054, XP002016243 IYER R.P. ET AL: "A Novel Phosporamidite Synthon Derived from 1R,2S-Ephedrine" see the whole document ---	1-26
X	JOURNAL OF THE CHEMICAL SOCIETY, PERKIN TRANSACTIONS 1, 1985, LETCHWORTH GB, pages 199-202, XP002016242 JONES A.S. ET AL: "Synthesis of some Nucleoside Cyclic Phosphoramidates and Related Compounds via Phosphoramidites" see page 199 ---	1,2,37

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 1996	21. 11. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Day, G

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF THE CHEMICAL SOCIETY, PERKIN TRANSACTIONS 1, 1993, LETCHWORTH GB, pages 1699-1704, XP002016241 KRASZEWSKI A. ET AL: "Studies on Reactions of Nucleoside H-Phosphonates with Bifunctional Reagents. Part 1. Reaction with Amino Alcohols" see the whole document	1,2,37
X	US 5 359 052 A (STEE WOJCIECH J ET AL) 25 October 1994 see the whole document	19,20
X	WO 92 00091 A (BIOLIGAND INC) 9 January 1992 see page 24, line 33 - page 26; claims	34-36
X	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 117, no. 11, 22 March 1995, DC US, pages 3302-3303, XP002018375 DEBENHAM J.S. ET AL: "Two New Orthogonal Amine Protecting Groups That Can Be Cleaved under Mild or Neutral Conditions" see the whole document	27,28
X	NL 8 700 724 A (TECHNISCHE UNIVERSITEIT EINDHOVEN) 17 October 1988 see figures	29
P,X	JOURNAL OF ORGANIC CHEMISTRY, vol. 60, December 1995, EASTON US, pages 8132-8133, XP002018376 IYER R.P. ET AL: "Methyl Phosphotriester Oligonucleotides: Facile Synthesis Using N-Pent-4-enoyl Nucleoside Phosphoramidites" see the whole document	27-31
P,X	JOURNAL OF ORGANIC CHEMISTRY, vol. 60, October 1995, EASTON US, pages 5388-5389, XP002016244 IYER R. P. ET AL: "Nucleoside oxazaphospholidines as Novel Synthons in Oligonucleotide Synthesis" see the whole document	1,9,10

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 96/07430

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		JP-T-	2503911	15-11-90
		WO-A-	8807542	06-10-88